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<b>13. ABSTRACT (Maximum 200 Words)</b> The estrogen receptor $\alpha$ (Era) plays an important role in breast cancer and a large fraction of Era positive breast cancers respond to tamoxifen. We cloned a novel p21 activated kinase (PAK), termed PAK6, which binds to the androgen receptor (AR) and selectively to the tamoxifen liganded Era. PAKs are a family of serine/threonine kinases that bind to and are regulated by the Rho family small (p21) GTPases, Cdc42 and Rac. PAKs are involved in translating extracellular signals into cellular responses. Although PAK6 binds to Cdc42, it lacks the Cdc42 regulated autoinhibitory domain founding other PAKs and can instead be activated by steroid receptor binding. Binding is mediated by at least two sites on PAK6, one at the N-terminus and another toward the middle of the protein. PAK6 inhibits Era and AR transcriptional activity. PAK6 is highly expressed in brain and testes, is also expressed in mammary epithelium and prostate, and its expression in breast cancer cell lines has been confirmed by a polyclonal antibody. Further studies of PAK6 protein expression in breast cancer are in progress, and breast cancer cell lines expression wildtype and mutant PAK6 have been generated to assess functions in breast cancer.				
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## INTRODUCTION

The classical estrogen receptor, estrogen receptor  $\alpha$  (ER $\alpha$ ) plays an important role in breast cancer development and a large fraction of ER $\alpha$  positive breast cancers respond to treatment with tamoxifen. We have cloned a novel p21 activated kinase (PAK), termed PAK6, which binds to the androgen receptor (AR) and selectively to the 4-hydroxytamoxifen (OHT) liganded ER $\alpha$ . PAKs are a family of serine/threonine kinases that bind to and are regulated by the active (GTP bound) form of the Rho family small (p21) GTPases, Cdc42 and Rac. PAK6 is expressed in normal mammary epithelium and in breast cancer cell lines, and its expression may be modified in breast cancer. Taken together, these results support a role for PAK6 in breast cancer. The purpose of this research is to test the major hypothesis that PAK6, through interactions with the ER $\alpha$ , contributes to the development of breast cancer. A second hypothesis to be tested is that the OHT dependent ER $\alpha$ -PAK6 interaction contributes to the therapeutic effect of OHT in breast cancer and the subsequent development of OHT resistance. The specific aims are 1) to determine the structure, cellular distribution, and activation state of endogenous PAK6 protein in normal breast and breast cancer cell lines; 2) to determine whether PAK6 contributes to ER $\alpha$  function and cell growth in breast cancer cell lines; and 3) to determine whether PAK6 contributes to tamoxifen sensitivity in breast cancer

## BODY

Over the past year we have made substantial progress on each of the aims. This progress is detailed below.

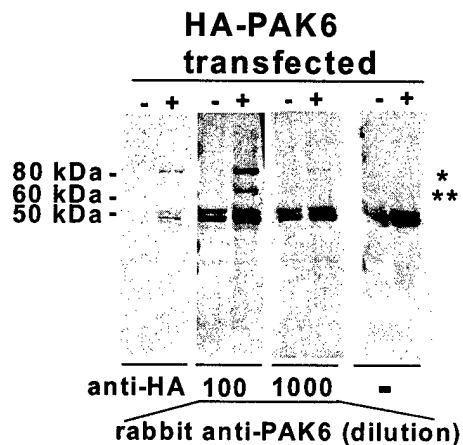
### **Task 1. Determine the structure, cellular distribution, and activation state of endogenous PAK6 protein in normal breast and breast cancer cell lines**

- a. Develop PAK6 antibodies (months 1-12)**
- b. Assess endogenous PAK6 using these antibodies (months 6-24)**
- c. Clone and sequence endogenous PAK6 from breast cancer cell lines (months 1-12)**

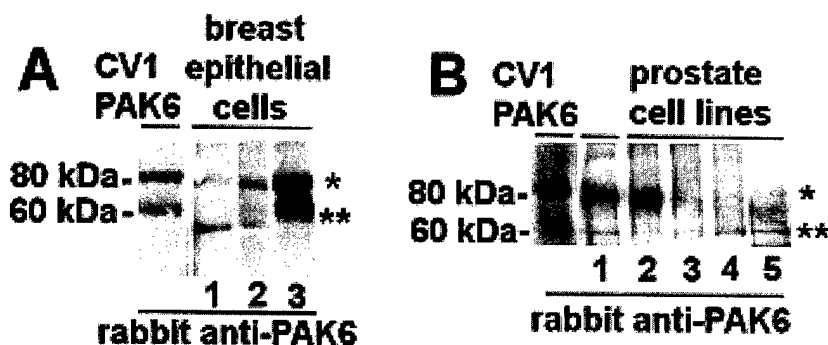
Rabbit polyclonal antibodies have been generated against PAK6 using a GST-PAK6 fusion protein. Over the past year we have affinity purified these antibodies and used them to detect and assess PAK6 expression. The PAK6 antibody was tested and titered by immunoblotting against lysates from CV1 and MCF7 cells transfected with HA epitope tagged PAK6 (tag at the N-terminus). The anti-PAK6 antibody used at approximately 250 ng/ml (1:100 dilution) readily detects a 75-80 kDa band that is identical to the band recognized by a monoclonal anti-HA antibody (Fig. 1). The anti-PAK6 also detects a second band at 65kDa that is not present in untransfected cells and is presumed to be a proteolytic product with the N-terminal removed (as it is not seen with the HA tag antibody). A doublet at about 50 kDa is recognized by the anti-rabbit secondary Ab (and to a lesser extent by the anti-mouse secondary) in MCF7 cells, but not other cell types. No further bands were detected, supporting the specificity of the PAK6 Ab.

Screens of breast cancer cell lines have revealed several that express high levels of PAK6, and functional studies are focusing on one of these lines (MDA-231) (Fig. 2). Preliminary studies of endogenous PAK6 kinase activity indicate that the kinase activity is strongly increased in the breast cancer cell lines. Current studies, including cloning and sequencing of the endogenous PAK6, are determining the basis for this activation. PAK6 was also expressed in each of the prostate cancer cell lines examined (LAPC4, LNCaP, CWR22Rv1, and PC3) (Fig. 2B). Immunohistochemical

analyses of normal breast and breast cancer are underway.



**Figure 1. Western blot analysis showing the specificity and titration of affinity purified anti-PAK6 antibody.** Lysates from MCF-7 cells transfected with HA tagged PAK6 are shown in + lanes and the untransfected in - lanes. The first panel is immunoblotting using mouse monoclonal HA antibody. The second and third are 1:100 (250 ng/ml) and 1:1000 (25ng/ml) dilutions of affinity purified antibody. The fourth is without primary antibody. Single or double asterixes indicate the intact PAK6 (\*) and a presumed proteolytic product (\*\*). The strong doublet at about 50 kDa is a protein in MCF7 that reacts with the secondary antibodies, which is not seen in most other cells examined.

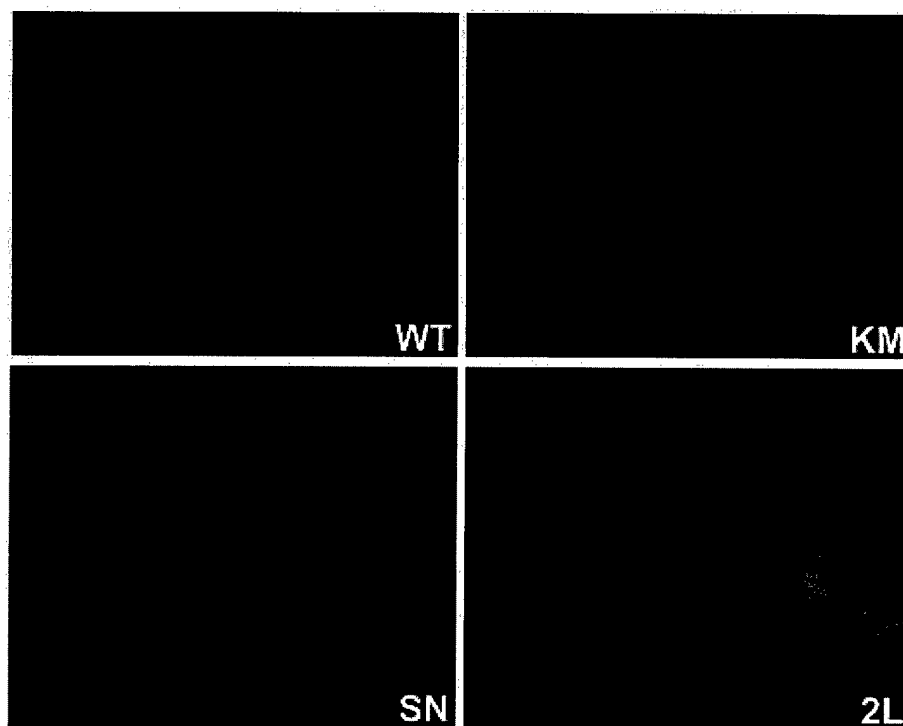


**Figure 2. Western blot analysis for PAK6 expression in breast and prostate cancer cell lines.** Equal amounts of protein (50 ug) from the indicated tumor cell lines, cells of PAK6 transfected CV1 cells were immunoblotted with anti-PAK6 (1:100 dilution). A, breast epithelial cells, Lane 1-HS578t, Lane 2-MDA MB231, Lane3-normal breast epithelial cells. B, prostate cancer cells, Lane1-MCF-7 (breast), Lane2-LAPC4, Lane3-LNCaP, Lane4-CWR22Rv1, Lane5-PC3.

**Task 2. Determine whether PAK6 contributes to ER $\alpha$  function and cell growth in breast cancer cell lines**

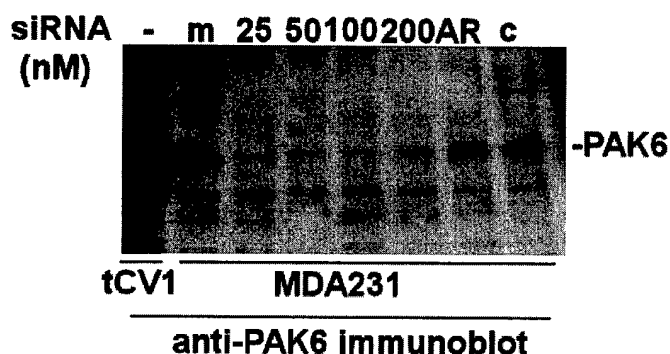
- a. Develop anti-sense methods to downregulate PAK6 (months 6-24)**
- b. Develop dominant negative PAK6 constructs (months 1-18)**
- c. Assess the functional consequences of blocking PAK6 (months 12-36)**

The initial efforts were to develop dominant negative PAK6 constructs. By site directed mutagenesis we generated vectors encoding PAK6 mutants that were constitutively active or kinase dead, as well as constructs that no longer bound Cdc42. LNCaP and MCF7 cell transfectants expressing these mutant proteins were generated and examined functionally. One result is that the Cdc42 binding mutant (2L) no longer associates with the plasma membrane (Fig. 3).



**Fig. 3. Immunofluorescence showing the localization of different mutants of PAK6 in LNCaP cells. WT-Wild type PAK; KM-Kinase dead, SN-Kinase active; 2L-CRIB domain mutant**

Over the past year we have generated small interfering RNA oligomers (siRNA) and have used these to markedly decrease expression of endogenous PAK6 with this siRNA (Fig. 4). We have also produced plasmids that express this siRNA and are now deriving cell lines with markedly decreased PAK6 expression. These lines are currently being derived for functional studies in MDA-231 cells, which express substantial endogenous PAK6. It is anticipated that these knockdown studies of the endogenous PAK6 will provide insights into function.



**Fig. 4. siRNA inhibition of PAK6 expression.** MDA231 breast cancer cells expressing endogenous PAK6 were transfected with a PAK6 siRNA pool (Dharmacon), at 25-200 nM as indicated. Controls included nontransfected (c), mock transfection (m), and transfection with androgen receptor siRNA (AR). The first lane is transfected PAK6, which runs slightly slower due to an N-terminal epitope tag.

**Task 3. Determine whether PAK6 contributes to tamoxifen sensitivity in breast cancer**

**a. Determine whether tamoxifen blocks PAK6 activation (months 18-30)**

**b. Determine whether the tamoxifen-ER $\alpha$ -PAK6 complex mediates cell cycle arrest (months 12-24)**

Over the past year we assessed effects PAK6 on tamoxifen induced apoptosis in MCF7 cells. The results have not yet revealed marked effects of PAK6, but they are ongoing and not yet conclusive.

**KEY RESEARCH ACCOMPLISHMENTS**

- PAK6 cloning
- demonstration of PAK6 interaction with ER $\alpha$  and AR
- identification of second binding site in PAK6 N-terminus
- generation of PAK6 antibodies
- affinity purification of PAK6 antibodies
- demonstration of PAK6 protein in breast and prostate cancer cells
- demonstration of PAK6 in normal and neoplastic breast epithelium
- generation of PAK6 mutants
- generation of MCF7 cell lines expressing increased levels of wild type and mutant PAK6
- generation of siRNA to downregulate PAK6 expression

**REPORTABLE OUTCOMES**

previous year:

manuscript: Lee et al., 2002 (appended)

cell lines: MCF7 cells expressing wild type or mutant PAK6

antibodies: polyclonal rabbit anti-PAK6 antibodies

current year:

affinity purified anti-PAK6 antibodies that can be used for immunohistochemistry  
siRNA that can block endogenous PAK6 expression

## **CONCLUSIONS**

During the previous period we completed and published our initial studies describing PAK6 and its interaction with the ER $\alpha$  and AR. We also generated a series of reagents that were needed to further address the functional significance of PAK6 and its interactions with steroid hormone receptors. Our major effort over the past year has been to affinity purify our antibodies and use them to assess PAK6 in normal and neoplastic breast, as well as breast cancer cell lines. The data show that PAK6 is indeed expressed, with increased expression and, significantly, increased activity, in a subset of tumors. The function of this PAK6 in breast cancer will continue to be the focus of our investigations.

## **REFERENCES**

Lee SR, Ramos SM, Ko A, Masiello D, Swanson KD, Lu ML, and Balk SP. 2002. AR and ER Interaction with a p21-Activated Kinase (PAK6). *Mol Endo* 16:85-99

## **APPENDICES**

none